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Effect of insulin treatment on circulating insulin-like growth factor (IGF)-I and IGF-binding proteins in cats with diabetes mellitus.

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Runnings head: Regulation of IGF-I in diabetic cats

Keywords: Insulin, IGF-II, ternary complex, IGFBP-3

Abbreviations:

ALS	Acid labile subunit
AUC	Area under the curve
DM	Diabetes mellitus

IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP 1-6	Insulin-like growth factor binding protein 1-6
MS	Mass spectrometry
ROC curves	Receiver operating characteristic curves
TC	Ternary complex
TT4	Total thyroxine

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Abstract

Background: Insulin-like growth factor-I (IGF-I) is used to screen for acromegaly in diabetic cats. In humans, most circulating IGF-I forms ternary complexes (TC) with IGF-binding protein (IGFBP-3) and an acid-labile subunit. Compared to humans, the amount of TC in cats is more variable. IGF-I concentrations are reported to increase during insulin treatment, more rapidly in cats achieving remission.

Objectives: To investigate (i) factors associated with circulating IGF-I concentrations, including IGF-binding protein profiles (ii) effect of insulin treatment on IGF-I concentrations and (ii) IGF-I as prognostic marker of DM remission.

Animals: 31 privately-owned diabetic cats of which 24 were followed 1 year, and 13 healthy cats.

Methods: In this prospective study, insulin, IGF-I, glucose and fructosamine were measured. IGF-binding forms were determined by chromatography in 14 diabetic and 13 healthy cats. IGF-I, IGF-II, IGFBP-3 and IGFBP-5 was measured by mass spectrometry in 3 cats achieving remission.

Results: IGF-I median (interquartile range) before start of insulin treatment was 300 (160-556) ng/mL. IGF-I was positively associated with TC ($p<0.0001$) and endogenous insulin ($p=0.005$) and negatively associated with fructosamine ($p<0.0001$). IGF-I was 2-fold higher 2-4 weeks after start of insulin treatment compared with baseline ($p=0.0001$) and predicted future remission ($p=0.046$). In cats that went into remission, the amount of TC and IGFBP-3 increased, suggesting the increase in IGF-I is dependent on TC formation.

Conclusions: Insulin treatment should be accounted for when interpreting IGF-I in diabetic cats. IGF-I 2-4 weeks after initiation of insulin treatment shows promise as prognostic marker for remission in diabetic cats.

Insulin-like growth factors (IGF-I and -II) and a family of six high affinity binding proteins (IGFBP-1 to -6) are important for growth and metabolism. In adult humans, >90% of IGFs are associated in a high affinity, GH-dependent, 150kDa complex with IGFBP-3 or IGFBP-5 and a third protein, the acid-labile subunit (ALS).[1,2] Formation of these ternary complexes (TC) prolongs the half-life of IGFs and is therefore a major determinant of circulating concentrations.[1] The stability of the complexes also makes IGF-I a good diagnostic marker of growth hormone excess. In adult humans, ALS circulates in excess of the TC and is therefore not a limiting factor for TC formation.[3] One study demonstrated that ALS was a limiting factor for TC formation in healthy cats, while in diabetic cats with high IGF-I concentrations, ALS apparently circulated in excess.[4]

Measurement of feline IGF-I is mainly used as a screening marker for acromegaly, which is almost exclusively diagnosed in cats with diabetes mellitus (DM).[5] Feline DM is considered to be similar to human type 2 DM and is characterized by insulin resistance and beta-cell failure.[6] The insulin-secreting capacity of feline beta-cells is blunted during hyperglycaemia[7] and insulin concentrations at diagnosis of DM are usually low.[8,9] Despite low insulin concentrations at diagnosis, cats can go into remission, defined as independence of insulin treatment $\geq$4 weeks.[10] Insulin concentrations at diagnosis do not predict remission.[10,11] Insulin treatment of feline DM has been observed to increase IGF-I[12,13] which normalized faster in cats achieving remission.[13] As in other species, feline IGF-I concentrations appear to be influenced by nutritional status.[14] Improved understanding of the IGF-system and the effect of insulin treatment is therefore essential for interpretation of IGF-I measurements in cats with DM.

The aim of this study was to further characterize the IGF-system in cats with DM by determining (i) factors associated with circulating IGF-I concentrations, including IGF-binding protein profiles (ii) the effect of insulin treatment on IGF-I concentrations and (ii) serum IGF-I as prognostic marker of DM remission.

Material and Methods

Animals

This study was approved by the Swedish Animal Ethics Committee and the Swedish Board of Agriculture (C301/10, C22/9, 31-105551/10 and 31-1364/09). All cat owners provided informed written consent. In total serum from 31 cats diagnosed with DM and 13 healthy cats was used (Fig 1). Cats were enrolled at Bagarmossen Animal Hospital in Stockholm, the University Animal Hospital in Uppsala and at four smaller clinics in Stockholm. Diagnosis of DM was based on clinical signs, hyperglycaemia >162 mg/dL detected in ≥2 evaluations and elevated fructosamine (>350 µmol/L). Cats were excluded from the study if they were pregnant or age <1 year.

For evaluation of IGF-binding profiles an initial blood sample was collected from 17 diabetic cats. In addition samples from healthy cats used for a previous study[15] were eligible for the laboratory work. These cats were considered healthy based on a questionnaire, physical exam and a basic biochemical profile (glucose, fructosamine, creatinine, ALT, albumin, protein). For IGF-binding profiles, 7 samples from cats with DM and 13 healthy cats were chosen to represent at range of IGF-I concentrations in both healthy and diabetic cats, thereby excluding 10 of the DM cats (Fig 1). IGF-I concentrations did not differ between excluded and included cats ($p=0.65$). Six of the cats with DM were sampled before insulin treatment and one cat was receiving insulin glargine.

Twenty-four cats diagnosed with DM but not yet on insulin treatment were enrolled in the longitudinal study. A management regime that included insulin treatment was required for inclusion. Cats were followed for a year from inclusion in the study or until remission, death or loss to follow up. Blood samples were taken five times: before first insulin injection (T0), after 2-4 weeks (T1), 8-10 weeks (T2), 5-7 months (T3) and 10-13 months (T4). At T0 cats were fasted

for at least 12 h, and at T1-T4 for at least 5 h. At T1-T4, all cats were given insulin q12h and blood sampling was performed by venipuncture into tubes without anticoagulant. The duration of effect of insulin glargine in cats is estimated to be approximately 11h.[16] Therefore, to minimize the interference of insulin glargine in the insulin assay, blood was taken just before the next q12h insulin injection. At every sampling occasion the veterinary practitioner performed a standardized clinical examination and completed a questionnaire. Body composition was evaluated by body condition score (BCS) and graded 1-5 with 1 being underweight and 5 overweight.[17] The treating veterinarian decided about additional diagnostic tests and thus not all cats had the same diagnostic-work-up.

Remission from DM was defined as no insulin treatment for one month, no clinical signs of DM and fructosamine concentrations within the reference interval (<350 µmol/L). Owners of cats that were euthanized during the study (n=4) or after (n=2) were asked for permission for post-mortem examination and all but one gave this permission. Post-mortem examinations were performed by the same board-certified pathologist. Early in the study period, CT with contrast enhancement of the pituitary gland was done in one cat with IGF-I >1000 ng/mL, when anaesthetized for dental treatment.

Analytical procedures

Blood samples were centrifuged within 30-60 minutes of sampling. Serum was aliquoted into two tubes. One aliquot was sent by post at ambient temperature for analysis of glucose and fructosamine at the Clinical Pathology Laboratory at the Swedish University Animal Hospital. Glucose was analyzed using hexokinase/glucose-6-phosphate dehydrogenase[a] and fructosamine by the nitrotetrazolium blue-method[b] using a standard biochemistry instrument.[c] The laboratory reported intra- and inter-assay CVs for glucose of 0.6 and 1.1%, respectively,

and for fructosamine 0.2 and 1.6%, respectively. The laboratory reported that serum glucose concentrations varied between -0.3 and 6.6% (n=7 samples) when stored at room temperature for two days and that fructosamine concentrations varied between -3.9 and 4.1% (n=3 samples) when stored at room temperature for three days. All samples arrived at the laboratory within two days.

The other serum aliquot was immediately frozen at -20°C, or kept at 2-4°C and frozen within 20h, and used for analysis of insulin and IGF-I. Feline insulin concentrations are stable for 4 days when stored at 2-4°C.[9] IGF-I concentrations in samples (n=2) stored at 2-4°C and analyzed after 3 and 6 days varied between -1.7 and 6.0% compared to baseline. IGF-I and insulin were measured with ELISAs[d,e] previously validated for use in cats.[9,15] There is a cross-reactivity of 8.4% for insulin glargine in the feline insulin ELISA.[18] Intra- and inter-assay CVs of the insulin assay were 2.0-5.8% and 7.6-13.7%, respectively. Intra- and inter-assay CVs of the IGF-I assay were 2.4-4.8% and 3.6-5%, respectively. For both ELISAs, multiple samples from the same cat were analyzed together on the same ELISA plate.

Size-exclusion chromatography

Size separation chromatography was performed on the single serum samples from healthy cats (n=13) and diabetic cats (n=7), as well as on samples from diabetic cats sampled at T0 and T1 (n=7). To visualize the IGF-binding protein profile in serum, samples were incubated with radioiodinated human IGF-II (^{125}I-IGF-II)[f] under neutral conditions, as previously described.[4,19] Tracer concentrations of ^{125}I-IGF-II, estimated <0.001% of total IGF concentration, were used to reflect the endogenous IGF-binding profile without disturbing the kinetics of the interactions between endogenous IGFs and IGF-binding proteins. Samples were therefore incubated until equilibrium was reached (17 h at 4°C). In total, 25 µL of serum (containing 50 000 cpm ^{125}I-IGF-

II, 1096 Ci/mmol, in a final volume of 100 µL of PBS (0.05 mol L^{-1}, pH 7.4) were loaded on a Superose 12 column[g] and eluted at 0.5 mL/min. Fractions were collected every 30 sec and radioactivity counted using a gamma counter.[h]

In another analysis serum was also size-fractionated, and fractions were analyzed for immunoreactive IGF-I using an IGF-I ELISA. This analysis was performed on serum from 2 healthy cats and one diabetic cat treated with insulin. The samples were diluted to 50% v/v with ammonium acetate (0.2 mol L^{-1}, pH 7.4). In total 100 µL serum in a final volume of 200 µL were size-separated under neutral conditions using ammonium acetate (0.2 mol L^{-1}, pH 7.4) as running buffer. Fractions were collected every 60 sec and dried by vacuum concentration. The fractions were analyzed with the IGF-I ELISA according to the manufacturer´s recommendation except that assay buffer was added directly to the dried fractions.

Mass spectrometry

A targeted mass spectrometry-based method (MS), previously validated for cats,[20] was applied to determine the concentrations of IGF-I, IGF-II, IGFBP-3 and IGFBP-5 in 3 cats with DM later achieving remission. Analyses were performed in serum at T0 and T1 exactly as described by Sundberg *et al*.[20] Briefly, the serum proteins were digested with trypsin and isotopically labelled internal standards, four QPrESTs[i] and one synthetic peptide[j], were added to the samples. The tryptic peptides were separated in reversed phase on an EASY-nLC 1000 system[k] and electrosprayed on-line to a QExactive Plus Orbitrap mass spectrometer[l] operating in parallel reaction monitoring (PRM) mode. The Skyline software[m] was applied for data analysis and quantification.

Statistical analysis

IGF-binding forms and concentrations of IGF-I, IGF-II, IGFBP-3 and IGFBP-5 measured by mass spectrometry

As previously described,[4] the amount of ^{125}I-IGF-II incorporated into the 150kDa molecular mass form was used as a marker for the relative amount of endogenous 150kDa-TC, while the 30-50kDa complex reflected binary forms. The 150kDa and 30-50kDa peaks were expressed as a 150/30-50kDa ratio which was estimated as a ratio of the sums of the five top-fractions for the 150kDa peak and 30-50kDa peak, respectively.

The association between IGF-I, age, sex, concurrent diseases and the relative amount of the 150kDa complex (the 150kDa/30-50kDa ratio) was evaluated by univariate linear regression analysis in a combined analysis of healthy cats (n=13) and diabetic cats before insulin treatment (n=13). Preliminary models showed non-normality of the residuals and the 150kDa/30-50kDa ratio was transformed to the natural logarithmic scale.

Differences between healthy and DM cats used for IGF-binding profiles were evaluated by Mann-Whitney test. In cats with DM sampled at T0 and T1, changes in the 150kDa/30-50kDa ratio as well as IGFBP-3, IGFBP-5 and IGF-II were evaluated by paired t-test after transformation to the natural logarithmic scale.

Predictors of IGF-I concentrations

To evaluate factors associated with IGF-I concentrations, a linear mixed-effect model was built using the statistical software R.[n] Potential predictors were age, concurrent diseases, days from T0, insulin, fructosamine, BCS and weight. Akaike Information Criteria and p-values were evaluated during modelling and predictors that did not contribute significantly to the model ($p>0.10$) were excluded. Residuals were evaluated for normal distribution, homoscedasticity

and linearity with respect to the predictors. Preliminary models showed non-normality of residuals and insulin and fructosamine were hence transformed to the natural logarithmic scale.

Predictors of remission

Predictors of remission were evaluated by univariate logistic regression and receiver operating characteristic (ROC) curves, to calculate area under the curve (AUC). Since remission of diabetes has been associated with tight glycaemic control,[21] glucose, fructosamine and insulin were considered potential predictors in addition to IGF-I. The statistical software R was used for logistic regression analyses.[n]

Results

IGF-binding forms and concentrations of IGFBP-3, IGFBP-5, IGF-II and IGF-I by mass spectrometry

Cats with DM used for evaluating IGF-binding forms were sampled once before insulin treatment and consisted of 2 castrated male cats, 3 castrated female cats and 1 intact male cat. Cats were between 8.2 and 20 years old and weighed between 3.9 and 6.3 kg. One cat had received glucocorticoids intratumoural in a cutaneous mast cell tumor before being diagnosed with DM and had renal insufficiency. One cat had cremor dentis and was obese, and two had urinary tract infection. No cat had elevated TT4. Glucose and fructosamine ranged from 19.5 to 33.3 mmol/L and 483 to 725 μmol/L respectively. Healthy cats consisted of 5 castrated males, 5 castrated females, 1 intact male and 2 intact females. Age ranged from 4.2 to 8.6 years and weight was between 3.0 and 7.1 kg. There was no significant difference in weight ($p=0.21$) or IGF-I concentrations ($p=0.68$) between healthy and DM cats used for size exclusion

chromatography, but DM cats were significantly older ($p<0.001$). Figure 2 shows the IGF-binding profiles in these 13 healthy cats and 6 diabetic cats sampled before insulin treatment, and presented for three equal IGF-I intervals generated based on a previously published reference interval for healthy cats (90-1207 ng/mL).[15] ^{125}I-IGF-II-tracer incorporated into 150kDa complexes reflect the proportion of endogenous IGFs circulating in a TC with IGFBP-3/5 and ALS, while relatively more tracer incorporation into 30-50kDa complexes represents the amount of endogenous binary complexes. In univariate linear regression analyses with IGF-I and ln-150/30kDa ratio, the regression lines showed no statistical differences in slope ($p=0.89$) or intercept ($p=0.29$) between healthy and diabetic cats, suggesting a similar IGF distribution across ternary and binary complexes between healthy and diabetic cats across the range of IGF-I concentrations. In a combined analysis with both healthy and diabetic cats the ln-150/30-50kDa ratio was correlated to IGF-I ($p<0.001$, R^2 52.4%)(Fig 3) but not to age ($p=0.83$), concurrent diseases ($p=0.48$) or sex ($p=0.57$).

Serum was also size-separated in the absence of ^{125}I-IGF-II and IGF-I immunoreactivity was determined by the ELISA in 2 healthy cats and in 1 cat on insulin treatment. In fractions where 150kDa and 30-50kDa ^{125}I-IGF-II-binding peaks elute, immunoreactive IGF-I was detected mainly in the 150kDa complex regardless of relative size of the 30-50kDa peak after size-separation with ^{125}I-IGF-II-tracer (data not shown).

Changes in IGF-binding profiles were determined in a longitudinal study. The largest increment in IGF-I concentrations was between T0 and T1 (Fig 4), therefore the distribution of IGF-binding forms at these two sampling occasions was investigated. At T0, the proportion of ^{125}I-IGF-II incorporated into the 150kDa complex was low in relation to the 30-50kDa complex both in cats that went into remission (Fig 5a) and in cats that did not go into remission (Fig 6a). During treatment, a relative increase of ^{125}I-IGF-II binding in the 150kDa complex was observed in the three cats that went into remission ($p=0.03$, Fig 5b) but not in those that did not achieve remission ($p=0.7$, Fig 6b). Concentrations of IGF-I, IGF-II, IGFBP-3 and IGFBP-5, all measured

by MS, are shown in Figure 5. In the three cats which went into remission, IGFBP-3 concentrations increased between T0 and T1 (p=0.03). IGF-II and IGFBP-5 did not differ between sampling occasions (p=0.4 and p=0.6, respectively). IGF-II was 2-8-fold higher than IGF-I. The molar ratio of IGFBP-3 and IGFBP-5 to total IGFs was 0.55 -1.1.

Cats included in the longitudinal study

Seventeen males (16 neutered and 1 intact) and 7 females (all ovariohysterectomised) were eligible for the longitudinal study. During the study period cats were followed for a year (n=11) or until remission (n=7), death (n=4) or loss to follow up at T2 or T4 (n=2) (Fig 1). Concurrent diseases were diagnosed in 13 cats and consisted of heart disease (n=2), hyperthyroidism (n=3), urinary tract infection (n=3) of which one cat also had tooth resorption, pancreatitis (n=2), chronic renal failure (n=1), osteoarthritis (n=1) and feline asthma (n=1). The cat with feline asthma was treated with fluticasone inhalation. In 18 of the 24 cats TT4 was measured at diagnosis of DM. In the remaining 6 cats TT4 was analyzed in samples taken at T0 and saved at -80°C and analyzed after study ending. One of these cats had high TT4 (64 nmol/L, reference interval 14-45 nmol/L) which was unrecognized during the study. Two cats that had previously been treated for DM, went into remission 15 and 26 months prior to study entry, and were diagnosed with DM again. Two cats were ketoacidotic and were initially treated with short-acting insulin intravenously. One of these cats was euthanized within 24h. Insulin glargine[m] was used in 22 cats and isophane insulin[n] in one cat. Owners of 15 cats used home monitoring with portable blood glucose meters. Veterinarians instructed owners to perform blood glucose curves at intervals varying from before every injection to 1 curve every 14 days. These 15 cats, and the other 9 cats, were also monitored by fructosamine and clinical signs. The surviving cat with ketoacidosis had been treated with glipizide but did not respond to treatment and had concurrent hyperthyroidism. This cat, and one additional cat with hyperthyroidism, were

receiving oral antithyroid medication (thiamazole). At T0, 2 cats were scored as underweight (BCS 1-2), 10 cats as normal weight (BCS 3) and 12 cats as overweight (BCS 4-5).

Descriptive statistics for the cats are presented in Table 1. IGF-I concentrations increased significantly between T0 and T1 (p=0.0001) but did not change significantly thereafter. Seven cats went into remission during the study period, all within five months from T0 (Fig 4). Of the 24 cats, eight (33%) had IGF-I concentrations >1000 ng/mL, a cut-off that is used when screening for acromegaly,[22,23] on at least one sampling occasion during the study. Five of the eight cats with IGF-I >1000 ng/mL achieved remission. Diagnostic imaging of the pituitary gland of one of these cats did not show any abnormalities and there were no clinical signs of acromegaly. Two additional cats with IGF-I >1000 ng/mL were subjected to post-mortem examinations with no findings suggestive of acromegaly. One female cat with IGF-I concentrations between 514 and 956 ng/mL had a pituitary adenoma on post-mortem examinations, however no immunohistochemistry was performed. This cat had no clinical signs of acromegaly noted by the veterinary practitioner and was on less than 0.2 IU/kg insulin q12h and was therefore not considered insulin resistant (defined as >1.5 IU/kg per dose).[24]

One cat sampled at T4 had serum insulin concentrations well above the other cats (Fig 4). At this sampling occasion the cat had a glucose concentration of 58 mg/dL, fructosamine 478 μmol/L and was on insulin glargine 0.6 IU/kg q12. The sample was reanalyzed twice with the same results.

Predictors of IGF-I concentrations

The mean increase in IGF-I between T0 and T1 was estimated to 350 ng/mL (95% CI 200-500). It was included as an offset in the model for predictors of IGF-I concentrations to account for possible selection bias caused by fallout due to remission. Weight was positively correlated and

fructosamine negatively correlated with IGF. However, fructosamine and weight were negatively correlated and we excluded weight because fructosamine was found to be a better predictor based on Akaike Information Criteria and p-values. As seen in Table 2, IGF-I concentrations were negatively associated with ln-fructosamine ($p<0.0001$) and positively associated with ln-insulin ($p=0.005$). The positive association between IGF-I and ln-insulin was linear up to an insulin concentration of 60 ng/L but no association was found at higher insulin concentrations. Age was not significantly associated with IGF-I ($p=0.052$) but improved the model fit and was kept in the model. Days from T0, BCS and concurrent diseases were not significantly associated with IGF-I and were excluded. In the final model, at insulin concentrations up to 60 ng/L, a doubling of insulin concentration was associated with an estimated increase in IGF-I of 95 ng/mL. An increase of fructosamine by 10% was associated with an estimated decrease of IGF-I by 47 ng/mL.

Predictors of remission

There was substantial overlap in IGF-I concentrations between groups at T0 and none of the biomarkers at T0 predicted remission. At T1, 22 diabetic cats remained in the study and of these six later went into remission. One cat achieved remission before T1 and was not available for the prediction model. IGF-I at T1 was associated with remission ($p=0.046$) but glucose, fructosamine and endogenous insulin were not ($p=0.11$, $p=0.24$ and 0.31, respectively). The estimated AUC in the ROC analysis for IGF-I was 0.80 (CI 0.62-0.99); for glucose 0.76 (CI 0.54-0.97); for fructosamine 0.65 (CI 0.39-0.90); and for insulin 0.61 (CI 0.33-0.90) (Fig 7).

Discussion

This study contributes to the understanding of the IGF system and its regulation in cats. Total IGF-I concentrations in serum were positively associated with the amount of IGF binding in a 150kDa TC, with lower IGF-I levels associated with relatively more binary IGF-IGFBP complexes. Furthermore, IGF-I concentrations were increased at 2-4 weeks after initiation of insulin treatment and higher IGF-I concentrations at this time-point were associated with remission from DM. An increase in circulating IGF-I with treatment was accompanied by a shift to increased amounts of TC compared to binding in lower molecular mass binary complexes. Consistent with this shift, IGFBP-3 concentrations were also observed to increase. It is therefore recommended that, when using IGF-I as a marker of GH status in cats with diabetes, the effect of insulin treatment on IGF-I should be taken into account.

IGF-binding forms

Most 150kDa complexes are formed of a high-affinity association between IGF-I or IGF-II and IGFBP-3 or IGFBP-5, and a lower-affinity association of ALS with these binary complexes.[25,26] The resulting TC cannot pass the endothelial barrier and are retained in the circulation, with a half-life of more than 12 h.[1] If sufficient ALS is available therefore, these TC contribute to the concentrations of IGF-I and IGF-II in the circulation. Under conditions of limited ALS availability, relatively more IGFs circulate in binary complex forms,[27] with a circulating half-life of hours.[1] This appears to be the case in cats with lower IGF-I concentrations. Therefore, we speculate that the wide variation in the amount of TC may be due to ALS not circulating in excess in many cats. The assumption has been made that in cats IGFBP-3 has a high affinity for IGF-I and IGF-II, similar to humans. While the affinity constants of feline IGFs and IGFBPs are not known, similar patterns of IGF-binding in the circulation are seen in other species.[28] In contrast to previously published results in cats where increased 150kDa/30-50kDa ratio was seen in

diabetic cats but not in healthy cats,[4] we saw a distribution of higher and lower molecular mass forms of IGF-binding in both healthy and diabetic cats. Furthermore, we observed that total IGF-I concentrations were positively associated with the relative amount of 150kDa complex. The discrepancy is explained by the fact that our population of cats, in contrast to the previous study, included a wide range of IGF-I concentrations.

When serum was size-separated and the fractions measured in an IGF-I ELISA, immunoreactivity was detected mainly in the 150kDa complex regardless of the amount of 30-50kDa complex. The ELISA measures only IGF-I whereas size-separation after adding iodinated IGF-II will reflect the amount of both IGF-I and IGF-II. Since IGF-I immunoreactivity was mainly found in the 150kDa form, and assuming the affinities and concentrations of IGFBPs are similar to other species, it is likely that the 30-50kDa peak reflects binary complexes with endogenous IGF-II. However, IGF-I and IGF-II affinities in the cat have not been studied and it is possible that radioiodinated human IGF-II has a different affinity for feline IGFBPs than feline IGF-II, which would affect interpretation of the results. Nevertheless, we chose to use the same methodology as previously used for studying feline IGF-binding forms.[4] Labeled human IGFs have been used for detection of feline IGFBPs on western ligand blots.[4,14,15] There is a report of similar finding of IGF-I immunoreactivity detected mainly in the 150kDa-peak, regardless the size of the 150kDa and 30-50kDa-peak, in children.[29]

IGF-I, IGF-II, IGFBP-3 and IGFBP-5 were also measured by mass spectrometry in three cats at baseline, and after diabetes remission. In these animals the increase in IGF-I was accompanied by an increase in IGFBP-3 concentrations. IGF-I and IGFBP-3 depend on each other for TC formation and stability in the circulation which prolongs their half-lives.[1] The increase in IGFBP-3 and IGF-I may be explained by an increase in ALS, or a direct stimulatory effect of insulin treatment on IGF-I and IGFBP-3 in the presence of sufficient ALS. As in humans,[30] IGFBP-5 in these cats circulated in lower concentrations than IGFBP-3. In humans binary complexes with IGFs and IGFBP-5 have a lower affinity for ALS than binary complexes

with IGFBP-3.[26] If feline IGFBP-5 binary complexes have a similarly lower affinity for ALS,[26] our results suggest it is unlikely that IGFBP-5 is a major contributor to TC formation in the cat. In the three animals studied, IGF-II concentrations did not increase during insulin treatment. We speculate that IGF-II may be an important component of binary complexes in cats and more readily available to tissues. Nevertheless, we recommend caution in extrapolating what is known about the IGF-physiology and pathophysiology between species.

Predictors of IGF-I during treatment

In the present study IGF-I concentrations were positively associated with insulin concentrations up to 60 ng/mL and negatively associated with fructosamine. In previous studies, weight has been associated with IGF-I in health and in diabetes.[14,15,31] In this study weight was excluded in the final model due to correlation with fructosamine and fructosamine gave a better model fit. The association between IGF-I concentrations, fructosamine and insulin needs to be taken into account when interpreting IGF-I concentrations and our findings support previous recommendations that screening for acromegaly in diabetic cats should be done after initiation of adequate therapy.[32]

There are likely multiple mechanisms underlying the association between insulin and IGF-I concentrations. Our results demonstrated a positive association at insulin concentrations up to 60 ng/L which was lost at higher insulin concentrations. Since hepatic GH-receptors will stimulate IGF-I synthesis one explanation could be an effect of insulin on hepatic GH-receptor expression.[33,34] One in vitro study found the effect of insulin on GH-receptor synthesis to be dose-dependent, declining at higher insulin concentrations.[33] Insulin stimulates ALS concentrations[35,36] and another possible explanation for the association between IGF-I and insulin is an effect of insulin on the circulating forms and therefore half-life of IGF-I. Like IGF-I,

ALS is GH dependent[1] and any effect on hepatic GH receptors may also play a role in regulating its synthesis.

One cat had very high insulin concentrations when sampled after a year of treatment with insulin glargine. The reason for the remarkably high insulin concentration was not determined. One possibility is interference of insulin antibodies towards insulin glargine, which could be measured in the insulin assay. In humans with DM a rare cause of hyperinsulinemic hypoglycemia is insulin antibodies.[37] Direct interference of insulin glargine in the assay is unlikely given a cross-reactivity of 8.4%, and taking into account the low dose of insulin glargine and sampling 12 hours after the injection.

Prediction of remission

Glycaemic control is considered the key factor for achieving remission in cats.[21,38] In the present study, high IGF-I concentrations 2-4 weeks after starting insulin treatment predicted remission of DM. Untreated DM may be regarded as a state of intracellular undernutrition. IGF-I concentrations are decreased in states of poor nutritional status in cats as well as in humans.[14,39] It is likely that initiation of insulin therapy increases IGF-I concentrations in part by an indirect effect, by promoting anabolism and reducing the catabolic state. It has been proposed that local IGF-I promotes survival of feline beta-cells and insulin production.[40] IGF-I may by enhancing insulin action[41] contribute directly to glycaemic control and therefore remission. The finding of increased serum IGF-I concentrations after initiation of insulin treatment in cats has been reported previously.[12,13]

Because glycaemic control is associated with increased remission rates[21] we decided to investigate glucose, fructosamine and insulin, in addition to IGF-I, as predictors of remission. None of these predictors reached statistical significance or the same AUC as IGF-I in the

statistical models. Cats can present with stress hyperglycaemia which may have affected glucose measurements in some of the cats,[42,43] and could explain why glucose did not perform better as a predictor. Fructosamine concentrations are dependent on protein turn-over time and blood glucose concentrations and are generally considered to represent glycaemic control during the last 1-2 weeks.[44,45] It is possible that fructosamine could be a predictor at later time points, however there were too few cats left in the study to explore this possibility. In agreement with previous reports,[10,11] insulin concentrations were not different between the two groups and are not useful in the prediction of DM remission in cats.

Implications for IGF-I as a screening tool for acromegaly

Although IGF-I concentrations were <1000 ng/mL in all cats at diagnosis, 33% of cats (8/24) reached levels >1000 ng/mL while on insulin treatment. This is a similar proportion to that reported in a study by Niessen et al.[23] None of the 8 cats with IGF-I >1000 ng/mL had clinical features of acromegaly and 5/8 cats went into remission, 2/8 cats had fructosamine concentrations within the reference interval and 1 cat was euthanized with no findings of acromegaly post mortem. However, in the study by Niessen at al[23] the majority of cats with DM and confirmed acromegaly did not present with phenotypical changes commonly associated with acromegaly making clinical features a less reliable diagnostic tool. In the present study, acromegaly was ~~only~~ excluded by diagnostic imaging or post mortem examination in 2/5 of cats with IGF-I >1000 ng/mL that went into remission from diabetes. Remission from diabetes is exclusively described in acromegalic cats after treatment with hypophysectomy[46] or pasireotide.[47] ~~.~~ Although we deemed it unlikely that the other three cats had acromegaly, ~~however~~ it is theoretically ~~be~~ possible~~acromegaly~~. ~~if insulin resistance was reduced and beta-cells were still viable.~~ In a recent study acromegaly was diagnosed in 3 cats without DM.[48] Different IGF-I immunoassays may not give the same concentration due to differences in type of

calibration material, immunoreactivity and extraction method for IGFBPs which otherwise interfere in the assay.[15,49] Given these observations we would suggest caution before using IGF-I values of 1000 ng/mL as a general cut-off for screening for acromegaly in diabetic cats.

Strengths and limitations

Reverse causation and selection bias were minimized by the longitudinal study design, which is a clear strength of this study. However, only about one-third of the cats went into remission and hence we had low power for the remission prediction models. We did not have access to an external validation cohort, which is a limitation because we may be overestimating the AUC. The reported remission rate of feline DM is 14-78%.[13,21,50-52] The remission rate in this study (29%) was in the lower range and may be due to inclusion of cats from both primary care and secondary care clinics, which may have differently motivated owners and differences in availability of specialists. The near-euglycaemic protocol has been associated with higher remission rates in cats[21] however the risk of hypoglycaemia may hold back both veterinarians and owners from using this approach. The treatment and investigations were decided by the treating veterinarians together with the cat owners. Therefore another limitation is that we did not perform the same diagnostic work-up in all cats. It is possible that some cats had underlying diseases not diagnosed which may affect IGF-I concentrations as well as IGF-binding profiles. However animals included in this study are likely to be representative of diabetic cats in general practice. Even though the statistical model did not show any significant effect of other diseases, only 13 cats with DM had concurrent diseases, making the statistical power low. It is also possible that any effect on IGF-I concentrations and IGF-binding profiles may vary with different diseases, however there were too few cats to perform these statistical calculations.

Conclusions

In conclusion, IGF-I concentrations increase after initiation of insulin treatment for feline DM and were associated with markers of glycaemic control. In cats that went into remission, the amount of 150kDa complex and IGFBP-3 increased, suggesting the increase in IGF-I is dependent on TC formation. The IGF-I concentration measured 2-4 weeks after initiation of insulin treatment shows promise as a predictive marker of remission from DM.

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Footnotes

[a] Glucose, Architect c Systems, Abbott Diagnostics, Illinois, US

[b] ABX Pentra, Horiba group, Montpellier, France

[c] Architecht c4000, Abbott Diagnostics, Illinois, US

[d] Feline Insulin ELISA, Mercodia, Uppsala, Sweden

[e] IGF-I ELISA, Mediagnost, Reutlingen, Germany

[f] ^{125}I-IGF-II, T-033-23, Phoenix Pharmaceuticals, CA, US

[g] Superose 12, 17-5173-01, GE Healthcare, Little Chalfont, UK

[h] Wallac Wizard- 2 2470, Perkin Elmer, Massachusetts, US

[i] QPrEST, Atlas antibodies, Stockholm, Sweden

[j] New England Peptide, Gardner, MA, US

[k] EASY-nLC 1000 system, ThermoFisher Scientific, Waltham, MA USA

[l] QExactive Plus Orbitrap mass spectrometer, ThermoFisher Scientific, Waltham, MA USA

[m] Skyline software, MacCoss Lab Software, University of Washington, Washington state, US

[n] The statistical software R. Vienna, Austria: R Foundation for Statistical Computing, URL http://www.R-project.org/; 2014: R Foundation for Statistical Computing

[m] Lantus, Sanofi AB, Paris, France.

[n] Insulatard, Novo Nordisk, Bagsvaerd, Denmark.

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Table 1. Descriptive statistics of 24 diabetic cats sampled during insulin treatment. Values are shown as median (interquartile range).

	Baseline (T0) (n=24)	2-4 weeks (T1) (n=22)	8-10 weeks (T2) (n=17)	5-7 months (T3) (n=14)	10-13 months (T4) (n=11)
IGF-I (ng/mL)	300 (160, 556)	670 (281, 1044)	562 (359, 926)	484 (306, 811)	444 (339, 743)
Fructosamine (µmol/L)	591 (545, 746)	524 (456, 607)	489 (340, 590)	520 (436, 578)	414 (286, 505)
Glucose (mg/dL)	414 (330, 501)	286 (182, 517)	265 (133, 434)	351 (223, 425)	105 (74, 382)
Insulin (ng/L)	42 (21, 81)	52 (30, 112)	85 (29, 226)	94 (35, 243)	39 (32, 547)
Weight (kg)	5.2 (4.3, 5.8)	5.4 (4.6, 6.0)	5.8 (4.7, 6.0)	5.8 (5.4, 6.5)	6.0 (5.0, 7.1)
Age (years)	11.0 (9.4, 12.7)	10.9 (9.3, 12.8)	10.9 (8.8, 12.4)	10.8 (8.9, 12.2)	11.3 (8.9, 12.2)
Insulin dosage (IU/kg)	N/A	0.3 (0.2, 0.4)	0.5 (0.2, 0.6)	0.4 (0.3, 0.6)	0.4 (0.3, 0.6)

N/A not applicable

Table 2. Linear mixed model analysis with IGF-I concentrations as the dependent variable in 24 cats with diabetes mellitus sampled up to five times during insulin treatment. The variance explained of the model (R^2adj) was 0.3.[1]

Predictor	β (95% CI)	P
Ln fructosamine	-489.5 (-670, -300)	<0.0001
Ln insulin	137.6* (44, 230)	0.005
Age (years)	36.3 (0, 73)	0.052

*The association was seen up to insulin concentrations of 60 ng/L

1. The variance explained of the model (R^2adj) was 0.3 disregarding the random effect for cat identity.

Legends

Figure 1. Flow-chart of study design.

Figure 2. IGF-binding profiles after size-separation in healthy and diabetic cats sampled before insulin treatment after incubation with human ^{125}I-IGF-II. Cats were grouped according to their IGF-I concentration analyzed with an IGF-I ELISA. Bars are presented as mean and standard error of the mean.

Figure 3. Scatterplot of 150/30-50kDa-ratio and IGF-I concentrations in 13 healthy (filled circles) and 13 diabetic cats sampled before insulin treatment (open circles).

Figure 4. Concentrations of IGF-I (A), fructosamine (B), insulin (C) and glucose (D) during the treatment of feline diabetes mellitus. Values are expressed as the median and interquartile range for 24 cats. Black circles with solid lines are cats which go into remission (T0: n=7, T1: n=6, T2: n=3) and open circles with broken lines are cats which do not go into remission (T0: n=17, T1: n=16, T2: n=14, T3: n=14, T4: n=11).

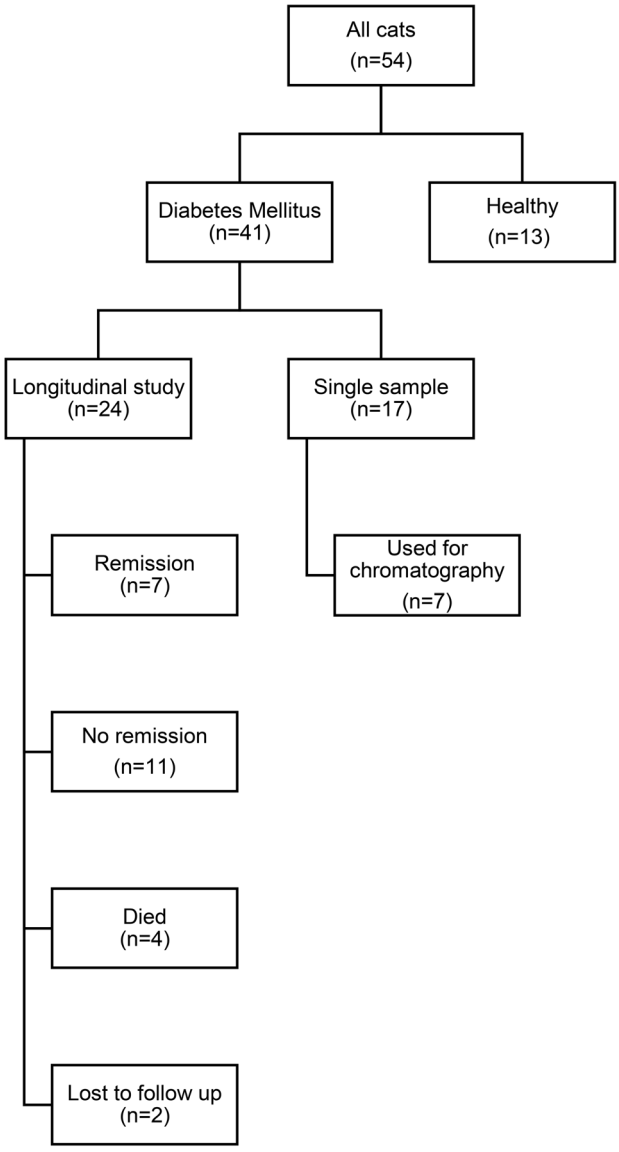
* Sample at remission is taken at least one month after insulin was withdrawn.

** One cat at T4 had insulin concentrations much higher than the others (7903 ng/L) and is not included in the graph.

Figure 5. Distribution of IGF-binding forms in three cats which went into remission, sampled before treatment, T0 (A) and at 2-4 weeks after insulin therapy, T1 (B). Serum was size separated on a Superose 12 column after incubated with human ^{125}I-IGF-II as described in material and methods. Results are expressed as counts per minute (C.P.M.). IGF-I,-II, IGFBP-3 and IGFBP-5 were measure by mass spectrometry.

Figure 6. Distribution of IGF-binding forms in four cats which did not achieve remission sampled before treatment, T0 (A) and at 2-4 weeks after insulin therapy, T1 (B). Serum was size separated on a Superose 12 column after incubated with human ^{125}I-IGF-II as described in material and methods. Results are expressed as counts per minute (C.P.M., mean $\pm$ SEM).

Figure 7. Receiver operating characteristic curves discriminating between cats going into remission or not. The curves were derived from 22 diabetic cats sampled 2-4 weeks after starting insulin treatment. Of these cats 6 went into remission. Area under the curve (AUC) and 95% confidence interval are indicated on the graphs.


All cats
(n=54)
Diabetes Mellitus
(n=41)
Healthy
(n=13)
Longitudinal study
(n=24)
Single sample
(n=17)
Remission
(n=7)
Used for
chromatography
(n=7)
No remission
(n=11)
Died
(n=4)
Lost to follow up
(n=2)